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Effects of the pine needle abortifacient, isocupressic acid, on bovine oocyte maturation and preimplantation embryo development

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Abstract

Isocupressic acid (ICA) [15-hydroxylabda-8 (17), 13E-dien-19-oic acid], a labdane diterpene acid, isolated from ponderosa pine (*Pinus ponderosa*), Lodgepole pine (*Pinus contorta*), common juniper (*Juniperus communis*) and Monterey cypress (*Cupressus macrocarpa*), induces abortion in pregnant cows when ingested primarily during the last trimester. The objective of this study was to investigate the effects of isocupressic acid on bovine oocyte maturation (in vitro maturation (IVM)—Experiment I) and preimplantation embryo development (in vitro culture (IVC)—Experiment II) using in vitro embryo production techniques and to subsequently evaluate viability and developmental competence of ICA-cultured embryos via embryo transfer to recipient heifers (Experiment III). A complete randomized block experimental design was used. In Experiment I and II, isocupressic acid was added to IVM or IVC media at 0 (TRT1, control), 1.3 (TRT2), and 2.6 μg/ml (TRT3) Results from Experiment I and II indicated that ICA did not inhibit oocyte maturation and did not adversely affect preinpiantation embryo development. Furthermore, results from Experiment II demonstrated that isocupressic acid enhanced bovine preimplantation embryo development in vitro in a dose dependent manner. Subsequently, Day 8 (Day 0 = IVF) blastocysts cultured in vitro in the medium containing 2.6 μg/ml ICA were transferred to recipient heifers and resulted in normal pregnancies as determined by ultrasound imaging. Subsequently, all but two births were normal as evaluated by post natal veterinary examination. In conclusion, ICA showed no adverse effects on oocyte maturation and preimplantation embryo development in vitro or subsequent viability in vivo using the ICA concentrations and in vitro culture parameters of this study.

Keywords: Bovine; In vitro fertilization; Isocupressic acid; Pine needles

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1. Introduction

Needles from ponderosa pine (*Pinus ponderosa*) induce abortion in pregnant cows when ingested primarily during the last trimester (James et al., 1989, 1994). Abortions have also occurred after natural ingestion or experimental feeding of lodgepole pine (*Pinus contorta*), common juniper (*Juniperus communis*) and Monterey cypress (*Cupressus macrocarpa*) (Gardner et al., 1998; Parton et al., 1996). The induced premature parturition often resulted in stillborn or small weak calves depending on the stage of gestation when cows ingested needles (James et al., 1989, 1994). Retained fetal membranes, endometritis and other reproductive complications are common. Direct losses from pine needle-induced abortion in the western US has been estimated to exceed $20 million annually (Miner et al., 1987; Nielsen et al., 1988).

One compound with abortifacient activity found in pine needles is isocupressic acid (ICA), as determined by oral feeding trials and intravenous infusions (Gardner et al., 1994, 1996, 1999). Isocupressic acid [15-hydroxylabda-8 (17), 13E-dien-19-oic acid], a labdane diterpene acid, was isolated from needles and bark of Ponderosa pine (*P. ponderosa*), Lodgepole pine needles (*P. contorta*), common juniper needles (*J. communis*) and Monterey cypress needles (*C. macrocarpa*) Abortifacient levels of ICA naturally occur in all of these species (Gardner et al., 1998). Incidence of abortion and time from pine needle ingestion to abortion is dependant on dosage of ICA ingested and/or stage of pregnancy. In feeding trials with pregnant cattle, cows fed a daily dose of 62–78 mg ICA/kg body weight of *P. contorta* aborted 8 to 10 days later. Cows daily fed *J. communis* at dosages of 190 and 245 mg ICA/kg body weight aborted after 3 and 4 days, respectively. All aborted cows had retained fetal membranes and classic clinical signs of pine needle-induced abortion (Gardner et al., 1994, 1996, 1998, 1999). Earlier feeding trials by Short et al. (1992) showed that the incidence of abortion in cows fed pine needles during different stages of gestation was increased as pregnancy progressed (Short et al., 1992).

The mechanism of action of isocupressic acid on bovine pregnancy is not fully understood. Ford et al. (1999) reported that *P. ponderosa* needle-induced abortion in late pregnant cows was related to a 56% decrease in uterine blood flow. They also reported that the plasma from pine needle-fed cows increased caruncular arterial tone (i.e., prolonged vasoconstriction) in an isolated bovine placentome perfusion system. The indirect vasoactive effects of pine needles on uterine blood flow may explain why the incidence of abortion in cattle increases with advancing pregnancy, i.e., a larger calf requires more oxygen and nutrients.

While Stegelmeler et al. (1996) described many of the abortifacient and pathological effects of isocupressic acid on reproduction, the effects of ICA on preimplantation embryo development that includes oocyte maturation, and embryo development through the morula and blastocyst stages have not been evaluated. The use of in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) techniques provides an analytical approach for obtaining information on the potential toxicological effects of chemicals during preimplantation embryogenesis (Bavister et al., 1992; Bavister, 1995; Cetica et al., 1999). By obtaining slaughterhouse ovaries, an abundant supply of oocytes for IVM, IVF and IVC were used for biological assessment and in vitro toxicological screening of ICA. The objective of

this study was to determine the effects of ICA on bovine preimplantation embryo development using in vitro embryo production techniques. Embryo transfer procedures were used to determine post implantation viability and developmental competence of ICA-exposed embryos.

2. Materials and methods

2.1. Production of bovine embryos in vitro

Bovine ovaries were collected from a local abattoir. Oocytes were aspirated from small antral follicles (3–8 mm in diameter) as described by Hawk and Wall (1994). Cumulus oocyte complexes (COCs) with evenly granulated ooplasm surrounded by several layers (at least three layers) of compact cumulus cells were selected for use according to the oocyte grading system of Hawk and Wall (1994). Oocytes were washed three times with Hepes-TALP solution (Parrish et al., 1988) and once with maturation medium. In vitro maturation of oocytes followed the procedure of Sirard et al. (1988) and Bavister et al. (1992) with minor modification. The maturation medium consisted of M-199 plus 10% (v/v) fetal bovine serum (FBS, A-1111, Hyclone Laboratories, Inc., Logan, UT, USA), 25 mM HEPES, 2 mM glutamine, 0.25 mM sodium pyruvate, 0.5 μg/ml ovine FSH (F-4520, Sigma Chemical Company, St. Louis, MO, USA), 5.0 μg/ml ovine LH (L-5269, Sigma) 1.0 μg/ml estradiol (E-2258, Sigma). Polystyrene plastic 4-well culture petri dishes (Nunclon®, Nunc Inc., Naperville, IL, USA) were used for IVM culture. Each well contained 500 μl IVM medium covered with paraffin oil (6358, Mallinckrodt Inc., Port, KY, USA). Approximately, 40–65 oocytes were transferred to the IVM medium per well and cultured in a humidified 5% CO atmosphere at 39 °C for 24 h. Cryopreserved bovine semen was used for in vitro fertilization. Live sperm were separated by Percoll (P-4937, Sigma) gradients (45 and 90% on the upper and lower layers, respectively) and centrifuged at 500 × g for 30 min. Motile spermatozoa were added to the fertilization medium (Fert-TALP, Parrish et al., 1988) to provide a final concentration of 2×10^6 cells per milliliter. Capacitation of spermatozoa occurred in Fert-TALP containing 10 μg heparin/ml and 0.6% (w/v) fatty acid free bovine serum albumin. IVM matured oocytes were added to Fert-TALP containing spermatozoa and cultured in plastic 4-well petri dishes under paraffin oil in a humidified 5% Co_2 atmosphere at 39 °C for 17 h. Each well contained 500 μl Fert-TALP and approximately 40–65 oocytes.

Cumulus and corona cells were removed from ova by vortexing in Hepes-TALP supplemented with 0.3% (w/v) bovine serum albumin for 3 min. The presumptive zygotes were then cultured in plastic 4-well petri dishes under paraffin oil at 39 °C in a humidified 5% Co_2 atmosphere. A modified CR2 medium (Wang et al., 1997) comprised of 108.3 mM NaCl, 2.9 mM KCl, 24.9 mM $NaHCO_3$, 2.5 mM hemicalcium lactate, 0.5 mM sodium pyruvate, BME amino acids (B-6766, Sigma), MEM nonessential amino acids (M-7145, Sigma), 0.5 mM glycine, 0.5 mM alanine, 1.0 mM glutamine, 1.0 mM glucose, and antibiotics was used to culture preimplantation embryos (IVC medium). Each well contained 500 μl IVC medium with approximately 35–55 oocytes. During culture, medium was changed every other day so that it contained 5% (v/v) fetal bovine serum on Day 1

(Day 0 = IVE), 10% on Day 3, 15% on Day 5, and 20% on Day 7 of culture (Zhang et al., 1992).

2.2. Embryo transfer

Recipient heifers were synchronized with Lutalyse® (Phamarcia & Upjohn Company, Kalamazoo, MI 49001, USA) following the manufacturer's instructions. Day 8 blastocysts derived from IVM/IVF/IVC techniques representing the highest concentration of ICA and non-ICA controls were non-surgically transferred to synchronized recipient females on Day 7 of their estrous cycle (two embryos/recipient). The recipients were checked for pregnancy by ultrasound at 35–40 days after embryo transfer. Pregnant recipients were maintained by routine animal husbandry methods until calving.

3. Experimental design

3.1. In vitro experiments

A complete randomized block experimental design was used to investigate the effects of ICA on bovine oocytes maturated in vitro (IVM medium—Experiment I), and in vitro culture of IVM/in vitro fertilization (IVF) derived embryos in IVC medium (Experiment II), respectively. Experiment I, IVM consisted of 24 replicates (total 4000 oocytes) and Experiment II, IVC consisted of 32 replications (total 4540 oocytes). In each of the replications, oocytes were from the same collection of abattoir ovaries. In each experiment, ICA of chromatographic purity (obtained from USDA-ARS, Poisonous Plant Research Lab, Logan, Utah, USA) was added to IVM or IVC media at 0 μg/ml (TRTl, control), 1.3 μg/ml (TRT2), and 2.6 μg/ml (TRT3). Oocyte cleavage rate was determined at 48 h after exposure of oocytes to spermatozoa. Embryo development was determined on Days 6, 8 and 10 of culture using an inverted microscope at 100 ×.

The ICA concentration of 1.3 μg/ml in the culture media was selected because it is considered a physiological level of ICA found in the blood of pregnant cows dosed with an abortifacient amount of pine needles (Gardner et al., 1999). The higher concentration selected was simply two times of the physiological concentration.

3.2. Embryo transfer (Experiment III)

The embryo transfer experiment was conducted consecutively for 2 years (Experiment III). Abattoir oocytes were matured and fertilized in vitro. The IVM/IVF derived embryos were cultured in media supplemented with either 0 μg/ml (control) or 2.6 μg/ml isocupressic acid (TRT). A random sample of morphologically normal blastocysts at Day 8 of IVC were taken from TRT and from control, respectively. Blastocysts were non-surgically transferred to synchronized recipient heifers on Day 7 of their estrous cycle (2 embryos/recipient). A total of 150 embryos and 75 recipient heifers were used (39 for treatment and 36 for control). Pregnancy rate and calving data were used to assess the viability and developmental potential of embryos from ICA and control treatments.

3.3. Statistical analysis

In Experiments I and II, percentage data were angularly transformed and analyzed by the use of a general linear model (GLM) ANOVA. The Fisher's least significant difference (LSD) at the 5% significant level ($P < 0.05$) was used to test the differences between treatment means. The differences between pregnancy and calving rates (Experiment III) were determined by the *t*-test. The number cruncher statistical system (NCSS 97) computer software package (Hintze, 1997) was used for all statistical calculations.

4. Results and discussion

The results from Experiment I demonstrate that bovine oocyte maturation is not adversely affected by ICA (Table 1). Percent development of morulae/blastocysts/expanded blastocysts in Table 1 was calculated with respect to cleaved oocytes. In vitro maturation of bovine oocytes in the presence of two concentrations of ICA did not alter oocyte maturation, and did not inhibit subsequent in vitro fertilization or embryo development ($P > 0.05$). Cleavage rates were 79.6, 78.9, and 80.4% and the percentage of morulae at Day 6 of IVC was 55.3, 56.7, and 55.4% for TRT1 (control), TRT2 and TRT3, respectively. The percentage of blastocysts at Day 8 of IVC was 26.3, 24.0, and 23.5% and percentage of expanded and hatched blastocysts at Day 10 was 22.0, 20.4, and 21.5% for TRT1 (control), TRT2 and TRT3, respectively. There was no significant ($P > 0.05$) difference with respect to oocyte cleavage rate or subsequent embryo growth and development between treatments when oocytes were cultured in the presence of ICA during maturation (Table 1).

Results from Experiment II indicate that the in vitro culture of bovine embryos in the presence of two concentrations of ICA did not adversely affect embryonic growth and

Table 1
The effects of isocupressic acid (ICA) on bovine oocyte maturation and preimplantation embryo development in vitro

Isocupresic acid concentration in medium (μg/ml)	Number of oocytes	Cleavage rate at 48 h[a] *n* (%)[b]	Morulae at Day 6[c] *n* (%)[d]	Blastocysts at Day 8 *n* (%)[d]	Expanded and hatched blastocysts at Day 10 *n* (%)[d]
Experiment i: isocupresssic acid added to IVM medium					
0 (TRT 1, control)	1357	1080 (79.6)	597 (55.3)	284 (26.3)	238 (22.0)
1.3 (TRT 2)	1346	1062 (78.9)	602 (56.7)	255 (24.0)	217 (20.4)
2.6 (TRT 3)	1297	1043 (80.4)	578 (55.4)	245 (23.5)	224 (21.5)
Experiment ii: isocupressic acid to IVC medium					
0 (TRT 1, control)	1526	1328 (87.0)	776 (58.4) a	285 (21.5) a	171 (12.9) a
1.3 (TRT 2)	1533	1309 (85.4)	837 (63.9) b	322 (24.6) b	214 (16.3) b
2.6(TRT 3)	1481	1279 (86.4)	796 (62.2) b	368 (28.8) b	266 (21.5) c

Values with different letters (a, b, c) are significantly different ($P < 0.05$).

[a] 0 h = the time when the in vitro matured oocytes were added to Fert-TALP containing spermatozoa.

[b] The percentage data were angularly transformed and analyzed by general linear model (GLM) ANOVA.

[c] Day 0 = IVF.

[d] Percent development of morulae/blastocysts/expanded blastocysts was calculated with respect to cleaved oocytes.

development. Interestingly, this data suggests that ICA promotes or enhances embryo development in vitro in a dose dependent manner. Not only is TRT 2 and 3 significantly ($P < 0.05$) better than TRT 1 (control) but TRT 3 is significantly ($P < 0.05$) better than TRT 2 when percentage of expanded and hatched blastocysts were compared, 12.9, 16.3 and 20.8% for TRT 1, 2 and 3, respectively. This data shows that there is no direct adverse effect from ICA on oocyte maturation, fertilization and cleavage rates or preimplantation embryo growth and development. Our observations are consistent with earlier anechdotal and field reports that non-pregnant cycling or early pregnant animals, including cattle, sheep, elk, deer, goats and rabbits are not adversely affected by pine needle ingestion (Panter et al., 1992; McEvoy et al., 2001). Data from this study supports earlier comparative studies in the investigation of the effects of feeding ponderosa pine needles at various stages of pregnancy showed that increased incidence of abortion in cattle occurred as later stages of pregnancy progressed. Short et al. (1992) fed pregnant cattle pine needles beginning from gestation Days 116–245. No abortions occurred after feeding cattle pine needles for 21 days beginning at 116 days of gestation. However, when pine needles were fed beginning on Days 167, 215 and 245, the incidence of abortion/early parturition increased with advancing pregnancy.

The data from this and other studies (McEvoy et al., 2001) indicate that ICA mainly interferes with late term pregnancy. The research we report here now extends the investigation of the effects of ponderosa pine needles and ICA on reproduction to the oocyte maturation and early embryonic developmental stages. This is supported by the data from Experiment III where ICA-exposed bovine embryos were transferred to recipient heifers. Pregnancy rates were 33.3 and 39.1% for ICA-treated embryos (2.6 μg/ml) and control, respectively. Ten calves were obtained from ICA-treated embryos and 8 calves from control. There was no difference ($P > 0.05$) between ICA and control (Table 2).

Although pregnancies progressed normally in Experiment III during years 1 and 2, two out of five calves derived from ICA-exposed embryos in the first year (Trial 1, Table 2) were stillborn. Upon necropsy, the stillborn calves had identical cardiovascular anomalies (large patent ductus arteriosis) that were considered the cause of death. The other three calves were born alive and developed normally to maturity. Physical examination and neonatal heart sounds in the three surviving calves at 2–4 weeks of age suggested that these animals

Table 2
Embryo transfer results (Experiment III) from IVF-derived bovine blastocysts cultured in the presence of isocupressic acid at 2.6 μg/ml in IVC medium

Treatment	Number of recipient heifers	Number of embryos transferred	Pregnancy rate at 35–40 days *n* (%)	Live calves born *n* (%)
Isocupressic acid 2.6 μg/ml in IVC medium				
Trial 1	24	48	8/24 (33.3)	5/48 (10.4)
Trial 2	15	30	NA	5/30 (16.7)
Total	39	78		10/78 (12.8)
Control no isocupressic acid in IVC medium				
Trial 1	23	46	9/23 (39.1)	4/46 (8.7)
Trial 2	13	26	NA	4/26 (15.4)
Total	36	72		8/72 (11.1)

did not have the cardiovascular problem. In the second year, all five calves obtained from ICA-treated embryos were normal and healthy at birth.

5. Conclusion

We conclude that the pine needle toxin ICA is not cytotoxic to bovine ooctyes or preimplantation embryos in vitro under the conditions of this experiment. While this research supports field observations and other studies that needles from the abortifacient trees, i.e. ponderosa pine, common juniper, lodgepole pine or Monterey cypress do not compromise early reproductive processes in cattle, further research in vivo is needed.

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